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Novel Cyclic Peptide, Epichlicin, from the Endophytic Fungus, *Epichloe typhina*

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The novel cyclic peptide, epichlicin, was isolated from *Epichloe typhina*, an endophytic fungus of the timothy plant (*Phleum pretense* L.). Its structure was determined by NMR studies and by mass spectrometry. Enantiomers of 3-amino tetradecanoic acid, a constituent amino acid of epichlicin, were synthesized as authentic standards. The stereochemistry of each amino acid was elucidated through a combination of the advanced Marfey method and chemical manipulation. Epichlicin showed inhibitory activity toward the spore germination of *Cladosporium phlei*, a pathogenic fungus of the timothy plant at an IC_{50} value of 22 nM.

Key words: endophyte; *Epichloe typhina*; *Cladosporium phlei*; germination inhibitor; cyclic peptide

Endophytes are microorganisms which infect and live symbiotically with host plants, and have been shown with a broad spectrum of host plants to induce strong resistance to some types of stress during their symbiosis. For instance, infection of barley (*Hordeum vulgare* L.) by the endophytic fungus, *Piriformospora indica*, induces resistance to fungal diseases caused by the necrotrophic fungus, *Fusarium culmorum*, and the biotrophic fungus, *Blumeria graminis*, and tolerance to salt stress, resulting in an overall increase in grain yield.[1)]

In the case of timothy (*Phleum pretense* L.), infection by the choke disease endophytic fungus, *Epichloe thyphina* (Pers. ex Fr.) Tul., whose immature form is *Acremonium typhinum*,[2)] induces resistance to leaf spot disease caused by the pathogen, *Cladosporium phlei* (Gregory) de Vries.[3)] A solution containing spores of *C. phlei* sprayed on to being timothy plants infected with *E. typhina* resulted in an aberration, a shortened germ tube length being identified in spore germination.[3)] Many antifungal compounds have been isolated from the choke, although these compounds have not been found in the aerial parts of timothy plants.[4–6)]

In order to explain this beneficial mutual relationship between the fungus and the host plant, we examined the metabolites of *E. typhina*, since a culture broth of *E. typhina* has been found to inhibit the spore germination in *C. phlei*.[3)] We therefore attempted to isolate the germination inhibitor from a culture broth of *E. typhina* to clarify the mechanism for the disease resistance induced by infection by the endophyte at the level of antifungal substances produced by the endophyte.

We report here the isolation and structural elucidation of the novel cyclic peptide, epichlicin (**1**), from a culture broth of *E. typhina*, and its biological activity as a spore germination inhibitor.

Material and Methods

General. FD-MS, FAB-MS and ESI-MS data were recorded with a JMS-SX102-A mass spectrometer (Jeol), and FAB-MS-MS data were recorded with a JMS-700TZ mass spectrometer (Jeol). NMR spectra were recorded with a JNM-EX 270 FT-NMR system (^{1}H at 270 MHz, Jeol) and an AM-500 NMR system (^{1}H at 500 MHz and ^{13}C at 125 MHz, Bruker). IR spectra were recorded with a 270-30 infrared spectrophotometer (Hitachi), and specific rotation values were determined with a DIP360 polarimeter (Jasco). Column chromatography was conducted with silica gel (Kanto Chemical), DIAION HP-20 (Mitsubishi Chemical), Sephadex LH-20 (Amersham Pharmacia Biotech), Develosil C_{30} (250 mm × 4.6 mm i.d., Nomura Chemical) and TSK gel ODS-80 Ts (150 mm × 4.6 mm i.d., Tosoh).

Incubation of Epichloe typhina. E. typhina was grown in 500-ml Erlenmeyer flasks which contained 200 ml of

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Abbreviations: TFA, trifluoro acetic acid; HPLC, high-performance liquid chromatography; FDLA, 1-fluoro-2,4-dinitorophenyl-5-L-leucinamide; RT, room temperature

a potato dextrose medium (200 g/l of potato, 10 g/l of peptone, 10 g/l of malt extract and 10 g/l of yeast extract). The fungus was grown in the dark for 50 days at 25 °C.

Germination inhibitory assay. C. phlei was incubated on a V-8 agar medium (V-8 juice, 200 ml/l; $CaCO_3$, 3 g/l; agar, 20 g/l) for 2–4 weeks at 25 °C. About 1 cm^2 of fungul mycelium was scraped off from the slant and suspended in 2 ml of water. The suspension was filtered through gauze, and the filtrate was used for a germination inhibitory assay. Five µl of the spore solution and 20 µl of each fraction (control; water) were mixed and pipetted on to a microscope slide. Each slide was put into a Petri dish and incubated at 25 °C for 12 h in the dark. After incubation, the percentage of germinated spores was calculated by comparing with the control.

Extraction and isolation. The isolation of the active compound was monitored with a germination inhibitory assay. The culture broth of *E. typhina* (1.7-liter) was partitioned with ethyl acetate (1.7-liter × 3). The water layer was subjected to DIAION HP-20 column chromatography (2-liter), the column being successively eluted with H_2O, MeOH:H_2O = 1:1 and MeOH. The MeOH eluent was concentrated *in vacuo* (560 mg), and then dissolved in MeOH. The soluble portion (200 mg) was subjected to Sephadex LH-20 column chromatography (70 g), using MeOH as the eluent. The active fraction (33 mg) was purified by HPLC (Develosil C_{30} column; CH_3CN:H_2O = 1:1; flow rate, 0.5 ml/min; A_{220}) to give 3 mg of compound **1** (t_R = 9.3 min, $[\alpha]_D^{25}$ +5.6°, *c* 0.33, MeOH). Epichlicin (**1**): IR ν_{max} (nujol): 3600–3300 (br.), 3300–3000 (br.), 1680 cm^{-1}. FAB-HR-MS *m/z* ($[M-H]^-$): calcd. for $C_{48}H_{73}N_{12}O_{14}$, 1041.5369; found, 1041.5338. ^{1}H- and ^{13}C-NMR spectral data are shown in Table 1.

Synthesis of (R) and (S)-3-amino tetradecanoic acids.

*Preparation of (E)-2-tetradecanoate (**3**) from dodecylaldehyde (**2**).* Dodecylaldehyde (**2**, 1.4 g, 6.5 mmol) and (*tert*-butoxycarbonylmethylene)triphenylphosphorane (2.8 g, 6.5 mmol) in CH_2Cl_2 (5 ml) were stirred in an argon atmosphere at room temperature for 12 h. After puriying by silica gel column chromatography (*n*-hexane: Et_2O = 19:1), *tert*-butyl (*E*)-2-tetradecenoate (**3**, 1.3 g, 4.6 mmol) was obtained in a 64% yield (E:Z = 96:4). FD-HR-MS *m/z* (M^+): calcd. for $C_{18}H_{34}O_2$, 282.2558; found, 282.2538. ^{1}H-NMR ($CDCl_3$) δ: 6.83 (1H, dt, J = 7.0, 15.4 Hz), 5.71 (1H, d, J = 15.4 Hz), 2.14 (2H, dt, J = 6.5, 7.0 Hz), 1.46 (9H, s), 1.40 (2H, m), 1.24 (16H, s), 0.86 (3H, t, J = 6.5 Hz). IR ν_{max} (film): 2928, 1872, 1720, 1040 cm^{-1}.

*Deprotection of compound **3**.* Compound **3** (1.3 g, 4.6 mmol) was dissolved in 3 ml of TFA and the solution stirred for 12 h at room temperature. The reaction mixture was evaporated to give tetradecenoate (**4**, 1.1 g) in a 99% yield. FD-HR-MS *m/z* (M^+): calcd. for $C_{14}H_{26}O_2$, 226.1932; found, 282.1922, ^{1}H-NMR ($CDCl_3$) δ: 7.07 (1H, dt, J = 6.8, 15.7 Hz), 5.80 (1H, d, J = 15.7 Hz), 2.21 (2H, dt, J = 6.8, 7.0 Hz), 1.45 (2H, t, 6.5 Hz), 1.25 (16H, s), 0.86 (3H, t, 6.5 Hz). IR ν_{max} (film): 3300–2500 (br.), 2956, 1696, 986 cm^{-1}.

*Synthesis of compounds **5**a and **5**b.* (*S*)-α-methylbenzylamine (270 mg, 2.2 mmol) was added to the solution of **4** (100 mg, 0.44 mmol) in 10 ml of pyridine, and the mixture was refluxed for 60 h. The reaction

Table 1. ^{1}H- and ^{13}C-NMR Spectra for Epichlicin (**1**) in CD_3OD

		δ_H[a]	δ_C[b]
Pro	α	4.25 (1H, t, 7.2 Hz)	63.2
	β	2.26 (1H, m)	30.2
	β	1.90 (1H, m)	
	γ	2.15 (1H, m)	26.3
	γ	2.00 (1H, m)	
	δ	3.94 (1H, m)	49.8
	δ	3.8 (1H, m)	
	C=O		175.2
Asn-1	α	4.62 (1H, m)	52.5
	β	2.75 (1H, dd, 7.9. 15.0 Hz)	36.7
	β	2.55 (1H, m)	
	C=O		173.1
	C=O[d]		174.6
Ser	α	4.39 (1H, dd, 3.8, 4.5 Hz)	57.6
	β	4.02 (1H, dd, 4.5, 12.0 Hz)	62.8
	β	3.78 (1H, dd, 3.8, 12.0 Hz)	
	C=O		172.7
Gln	α	4.68 (1H, dd, 4.5, 10.0 Hz)	51.7
	β	2.15 (1H, m)	27.7
	β	2.00 (1H, m)	
	γ	2.32 (2H, t, 6.2 Hz)	31.8
	C=O		172.8
	C=O[d]		177.7
Asn-2	α	4.53 (1H, dd, 4.5, 7.0 Hz)	51.7
	β	2.93 (2H, m)	36.4
	C=O		173.2
	C=O[d]		175.2
Tyr	α	4.32 (1H, dd, 4.0, 9.0 Hz)	57.6
	β	3.10 (1H, dd, 4.0, 14 Hz)	36.7
	β	2.91 (1H, m)	
	Bz-*o*	7.06 (2H, d, 8.4 Hz)	131.2
	Bz-*m*	6.72 (2H, d, 8.4 Hz)	116.2
	Bz-*I*		128.9
	Bz-*p*		157.3
	C=O		174.2
Asn-3	α	4.59 (1H, m)	52.5
	β	2.68 (1H, m)	37.8
	β	2.50 (1H, m)	
	C=O		174.6
	C=O[d]		174.7
β-AA[c]	α	2.40 (1H, d, 15.5 Hz)	43.5
	α	2.55 (1H, dd, 10.8, 15.5 Hz)	
	β	4.18 (1H, m)	48.1
	γ	1.59 (1H, m)	36.3
	γ	1.47 (1H, m)	
	$9CH_2$	1.23–1.35 (18H, m)	33.1, 30.6, 27.0, 23.7[e]
	CH_3	0.89 (3H, t, 6.9 Hz)	14.4
	C=O		174.5

[a] ^{1}H-NMR at 500 MHz referenced to CD_3OD (δ 3.30)
[b] ^{13}C-NMR at 125 MHz referenced to CD_3OD (δ 49.0)
[c] β-AA, β-amino acid
[d] C=O, γC=O of Asn residue and δC=O of Gln residue
[e] Some signals overlapped.

mixture was successively washed with 1 M HCl and water, and the organic layer was then concentrated *in vacuo* and purified by silica gel column chromatography (MeOH:$CHCl_3$ = 3:17). Two diastereomers, **5**a (20 mg, 0.057 mmol, 13%, ≧99% d.e.) and **5**b (12 mg, 0.036 mmol, 8%, ≧99% d.e.) were obtained. (3*S*, 1′*S*)-3-(1-Phenyl ethylamino)tetradecanoic acid (**5**a) was characterized as follows. EI-HR-MS *m/z* (M^+): calcd. for $C_{22}H_{37}NO_2$, 347.2824; found, 347.2811. $[\alpha]_D^{25}$ +16.3° (*c* 0.16, MeOH), ^{1}H-NMR ($CDCl_3$) *δ*: 7.50–7.25 (5H, m), 4.18 (H-*α*, 1H, q, *J* = 6.8 Hz), 2.87 (1H, br.s), 2.58 (H-*β*, 1H, dd, *J* = 4.1, 16.2 Hz) 2.30, (H-*β*, 1H, dd, *J* = 4.1, 16.2 Hz), 1.66 (3H, d, *J* = 6.8 Hz), 1.40–1.10 (20H, m), 0.85 (3H, t, *J* = 6.8 Hz). IR (film): 3300–2500, 2928, 1720 cm^{-1}. (3*R*, 1′*S*)-3-(1-phenyl ethylamino)tetradecanoic acid (**5**b) was characterized as follows. EI-HR-MS *m/z* (M^+): calcd. for $C_{22}H_{37}NO_2$, 347.2824; found, 347.2808. $[\alpha]_D^{25}$ −25.4° (*c* 0.13, MeOH). ^{1}H-NMR ($CDCl_3$) *δ*: 7.42–7.29 (5H, m), 4.31 (1H, q, *J* = 6.8 Hz), 2.86 (1H, br. s), 2.47 (2H, br. s), 1.71 (3H, d, *J* = 6.8 Hz), 1.20–1.10 (20H, m), 0.86 (3H, t, *J* = 6.8 Hz). IR (film): 3300–2500 (br.), 2928, 1720 cm^{-1}.

Hydrogeneration of compounds 5a and 5b. Compounds **5**a (20 mg, 0.057 mmol) and **5**b (12 mg, 0.036 mmol) were separately dissolved in 3 ml of acetic acid and hydrogenated for 3 h under H_2 catalyzed by Pd/C. Each reaction mixture was filtered through Celite and purified by silica gel column chromatography (MeOH:$CHCl_3$ = 2:3) to give the desired *β*-amino acids, **6**a (12 mg, 0.047 mmol, 83%, ≧99% e.e.) from **5**a and **6**b (5.2 mg, 0.021 mmol, 60.1%, ≧99% e.e.) from **5**b. (3*S*)-Amino tetradecanoic acid (**6**a) was characterized as follows. FAB-HR-MS *m/z* ($M-H)^-$: calcd. for $C_{14}H_{28}NO_2$, 242.2120; found, 242.2126. $[\alpha]_D^{25}$ +16.0° (*c* 0.90, H_2O). ^{1}H-NMR (CD_3OD) *δ*: 2.48 (1H, dd, *J* = 4.1, 16.5 Hz), 2.27 (1H, dd, *J* = 8.9, 16.5 Hz), 1.61 (2H, m), 1.29 (20H, s), 0.89 (3H, t, *J* = 6.8 Hz). IR (film): 3300–2500 (br.), 2924, 1720 cm^{-1}. (3*R*)-Amino tetradecanoic acid (**6**b) was characterized as follows. FAB-HR-MS *m/z* ($M-H)^-$: Calcd. for $C_{14}H_{28}NO_2$, 242.2120; found, 242.2126. $[\alpha]_D^{25}$ −15.3° (*c* 0.90, H_2O). ^{1}H-NMR (CD_3OD) *δ*: 2.48 (1H, dd, *J* = 4.1, 16.5 Hz), 2.27 (1H, dd, *J* = 8.9, 16.5 Hz), 1.61 (2H, m), 1.29 (20H, s), 0.89 (3H, t, *J* = 6.8 Hz). IR (film) 3300–2500 (br), 2924, 1720 cm^{-1}.

Determination of the stereochemistry of compound 1 by the advanced Marfey method. Compound **1** (500 μg) was hydrolyzed in 1 ml of 6 M HCl at 120 °C for 12 h. The reaction product was dissolved in 50 ml of water and 20 ml of 1 M $NaHCO_3$, and reacted with 100 μl of the advanced Marfey reagent, FDLA (1 mg/100 ml in acetone), at 45 °C for 1 h. The reaction was stopped by adding 20 μl of 1 M HCl, and the mixture diluted with 810 μl of acetonitrile. To determine the stereochemistry of *α*-amino acid, the sample was analyzed by reverse-phase HPLC in a C_{18} column (Tosoh TSK-gel 80Ts), using a linear gradient of 15–45% CH_3CN in 0.1% aqueous TFA for 45 min at a flow rate of 1.0 ml/min. The UV absorbance was recorded at a wavelength of 340 nm. The retention times (min) of the FDLA derivatives of the hydrolysate of compound **1** were as follows (standard figure in brackets): L-Ser; 19.4 (19.3); L-Asp, 20.4 (20.3); L-Glu, 22.8 (22.8); L-Pro, 27.7 (27.8); D-Tyr, 37.2 (37.1). The *β*-amino acid was analyzed by using another solvent system (MeOH:0.1% aqueous TFA = 9:1, 1.0 ml/min). The retention times of FDLA derivatives of the standards of the (*S*)- and (*R*)-*β*-amino acids were 5.5 and 9.7 min, respectively, and that of the hydrolysate of compound **1** was 9.5 min.

Results and Discussion

Isolation and planar structure of compound 1

Isolation of the germination inhibitory compound was monitored by a germination inhibitor assay. The culture broth of *E. typhina* was purified by DIAION HP-20, Sephadex LH-20 and reverse-phase HPLC with a C_{30} column to give compound **1**.

Compound **1** was isolated as a colorless powder. The molecular formula of compound **1** was established as $C_{48}H_{74}N_{12}O_{14}$ by the FAB-HR-MS data. The ^{13}C-NMR data together with the molecular formula showed that compound **1** had 12 amide-type carbonyls. The ^{1}H-NMR data showed many peaks around *δ*2.00–4.00. The ^{1}H- and ^{13}C-NMR spectra of compound **1** showed close similarity to those of mixirin A, a cyclic peptide, which has been isolated from marine *Bacillus* sp.[7] The COSY data showed the presence of one Ser, one Pro, one Gln, one Tyr, three Asn residues and a residue having a long alkyl chain. Further analyses of COSY, HMQC and HMBC data revealed this residue to be 3-amino tetradecanoic acid (*β*-amino acid). The presence of 17 exchangeable protons was shown by ESI-MS data measured after dissolution in CD_3OD, showing an $[M+Na+17]^+$ ion peak at *m/z* 1082, supporting the predicted amino acid composition. As a result, the constituent amino acids of compound **1** were determined to be the same as those of mixirin A.[7]

The HMBC spectrum showed correlation between *α*-hydrogen of each amino acid and the carbonyl carbon of the adjacent amino acid (Fig. 1). An analysis of the fragmentation of compound **1** obtained from the FAB-MS-MS data supported the amino acid sequence determined from the HMBC spectrum (Fig. 1). Accordingly, the planar structure of compound **1** was determined to be identical to that of mixirin A (Fig. 1), although the specific rotation value differed from that of mixirin A (compound **1**, $[\alpha]_D^{25}$ +5.6°; mixirin A, $[\alpha]_D^{25}$ −18.2°). We therefore expected that epichlicin was a diastereoisomer of mixirin A.

Synthesis of (R)- and (S)-3-amino tetradecanoic acid

Compound **1** contained a *β*-amino acid as a constituent. To determine the stereochemistry of this residue, (*S*)- and (*R*)-3-amino tetradecanoic acids were synthe-

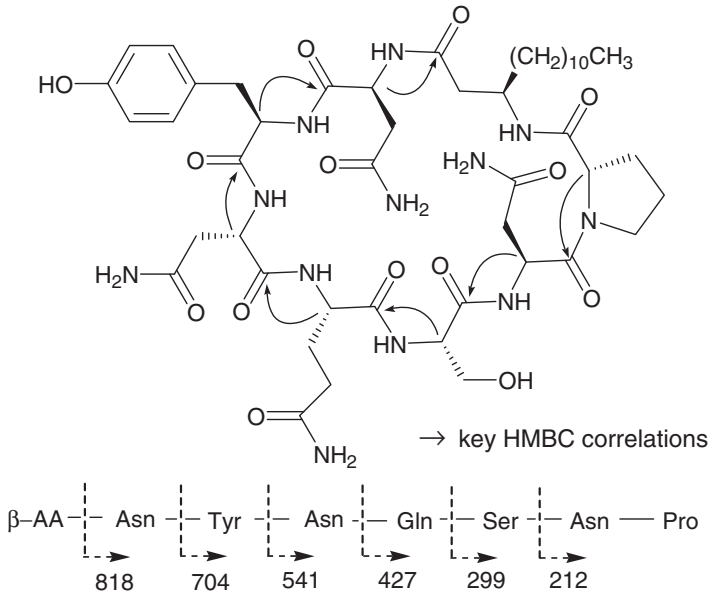
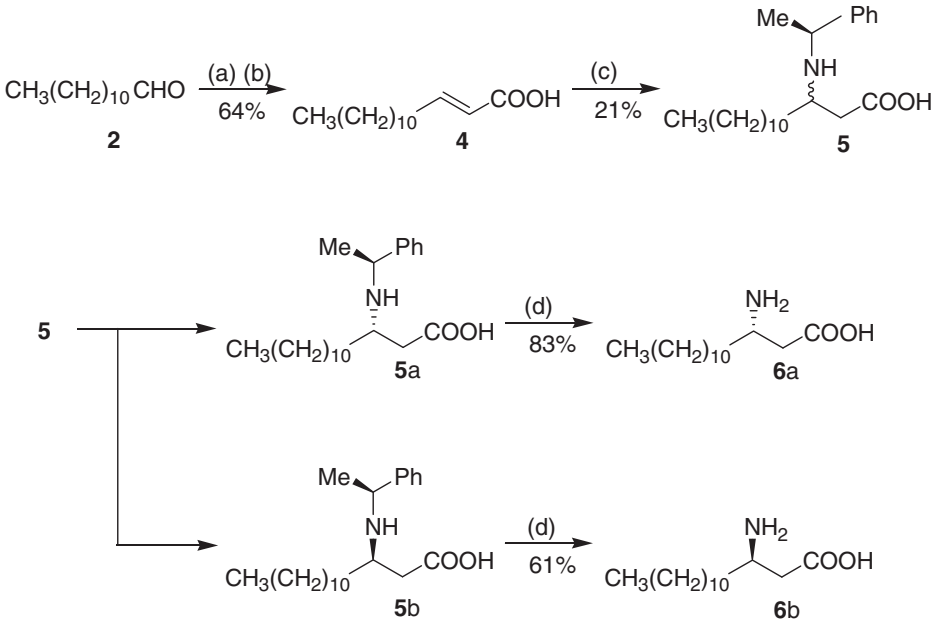


Fig. 1. Absolute Structure of Epichlicin (**1**) and the Fragmentation Pattern of Compound **1** Obtained from FAB MS-MS Data (positive ion mode). β-AA, β-amino acid



Scheme 1. Synthesis of (*S*)- and (*R*)-3-Amino Tetradecanoic Acid.
Reagents and conditions: (a) (*tert*-butoxycarbonylmethylene)triphenylphosphorane, CH_2Cl_2, RT, 12 h; (b) TFA, RT, 12 h; (c) (*S*)-α-methylbenzylamine, pyridine, reflux for 60 h; (d) Pd/C, AcOH, H_2, 50–60 °C, 5 h.

sized as authentic standards as shown in Scheme 1. Starting with dodecyl aldehyde (**2**), an α, β-unsaturated ester (**3**) was obtained by the Wittig reaction in a 69% yield. After deprotecting the *tert*-butyl group in TFA, α, β-unsaturated carboxylic acid (**4**) was reacted with (*S*)-α-methyl benzylamine (Aldrich, enantiomeric ratio $\geqq$99.5%) to give two diastereoisomers. These were separated by silica gel column chromatography (**5**a, 13%; **5**b, 8%). The diastereomeric excesses of **5**a and **5**b were both calculated to be 99% on the basis of the ^{1}H-NMR signals for H-α and H-β of **5**a and **5**b. Each of the diastereoisomers was separately hydrogenated in acetic acid under an H_2 atmosphere catalyzed by Pd/C to remove the benzyl group, giving the desired compounds (**6**a, 83%; **6**b, 61%). The enantionmeric excesses of **6**a and **6**b were both estimated to be $\geqq$99% on the basis of the diastereomeric excess of **5**a and **5**b (reported data, e.e. = 93%[8]). The specific rotation value of (*S*)-3-amino tetradecanoic acid has been reported as +28.8° (*c* 0.90, H_2O, 25 °C),[8] the specific rotations values of **6**a

and **6**b being +16.0° (*c* 0.90, H_2O, 25 °C) and −15.3° (*c* 0.90, H_2O, 25 °C), respectively. Thus, the stereochemistry of **6**a was determined to be (*S*), and that of **6**b to be (*R*). The reported specific rotation value is thought to be in error, and this may be responsible for the difference between the reported and our data. Although the enantioselective synthesis of 3-amino tetradecanoic acid has been reported,[8] the synthesis of these compounds *via* diastereomers is novel to our knowledge.

*Determination of the absolute stereochemistry of compound **1***

The stereochemistry of each amino acid in mixirin A has been determined by Marfey's method as D-Pro, D-Tyr, L-Ser, L-Asn and L-Gln,[7] although the stereochemistry of the β-amino acid has not been shown. We therefore determined the stereochemistry of each amino acid, including the β-amino acid, with the advanced Marfey method.[9,10]

Compound **1** was hydrolyzed in 6 M HCl, and then reacted with the advanced Marfey reagent, FDLA.[9,10] After its conversion with FDLA, the sample was analyzed by reverse-phase HPLC. The stereochemistry of the α-amino acids was determined as L-Pro, D-Tyr, L-Ser, L-Asn and L-Gln, respectively. Although the stereochemistry of the proline of mixirin A has been reported to be D, that of epichlicin was confirmed as L. An analysis of the β-amino acid was made by using a different solvent system. The retention times of the FDLA derivatives of the synthesized (*S*)- and (*R*)-β-amino acids were 5.5 and 9.7 min, respectively. The peak of the FDLA derivative of the hydrolysate of compound **1** appeared at 9.5 min, enabling the stereochemistry of the β-amino acid to be determined as having (*R*) configuration (**6**b in Scheme 1). In conclusion, the total absolute stereochemistry of compound **1** was determined to be that shown in Fig. 1. The stereochemistry of the proline of epichlicin, differing from that of mixirin A, and the stereochemistry of the β-amino acid, which had not been determined in mixirin A, was found to be (*R*). We named compound **1** as epichlicin.

Germination inhibitory activity of Epichlicin against C. phlei

Epichlicin (**1**) showed germination inhibitory activity against *C. phlei* at an IC_{50} value of 22 nM (Fig. 2). This test was conducted three times and the reproducibility was confirmed. Epichlicin also inhibited germ tube elongation of *C. phlei* at 20 and 32 nM (Table 2).

Under normal conditions, *E. typhina* produces epichlicin at around 2 μM level in the rapid-growth stage. The inhibitory effect of epichlicin against *E. typhina* is therefore much less than that against *C. phlei*.

As previously mentioned, infection by *E. typhina* of the timothy plant induces resistance against *C. phlei*. There are also some reports about the host plant obtaining resistance against some types of stress through infection by the endophyte. Our results indicate the possibility that some organic compounds produced by the endophyte play an important role in this induced resistance. Epichlicin may thus be useful in defending plants from disease stress.

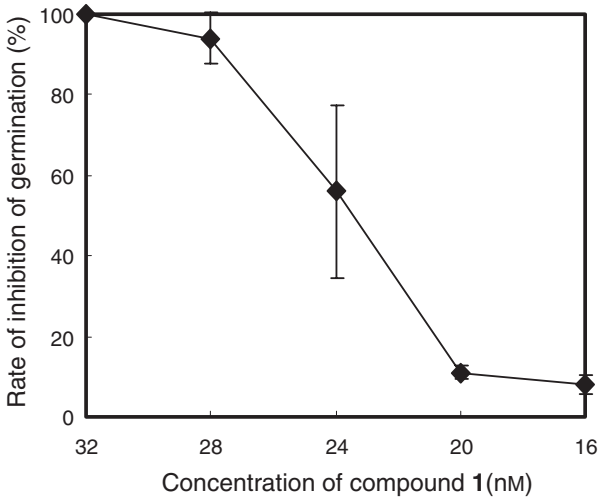


Fig. 2. Germination Inhibitory Activity of Epichlicin (**1**) against *C. phlei*.

Each value is expressed as the mean ± standard deviation from three results.

Table 2. Germ Tube Elongation Inhibitory Activity of Epichlicin against *C. phlei*

Five μl of the spore solution was pipetted on to a microspore slide and incubated for 12 h. Then, 20 μl of a solution of epichlicin was added (control, water) and incubated for more 12 h. After incubating the length of the germ tube was measured. Each value is the average ± SD (n = 10).

Concentration of epichlicin (nM)	32	20	Control (0)
Length of germ tube (mm)	0.16 (±0.031)	0.20 (±0.026)	0.25 (±0.037)

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